

2 copies of Toxicity & Teratogenicity Studies in Avian Embryos-Butylated Hydroxy
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BUTYLATED HYDROXY ANISOLE

TOXICITY and TERATOGENICITY STUDIES
in Avian Embryos

FDA Contract #71-330

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STUDIES on the TOXICITY and TERATOGENICITY
of BUTYLATED HYDROXY ANISOLE in AVIAN EMBRYOS

SUMMARY and CONCLUSION

Butylated hydroxy anisole was toxic to chicken embryos in each of the four test protocols. LD-50 estimates indicate that this compound was more highly toxic to 96 hour embryos than when injected into the fertilized egg prior to incubation.

This compound produced hypopigmentation of the down of hatched chicks when injected in either the air cell or yolk prior to incubation, indicating an impairment of carotenoid (xanthophyll) metabolism or deposition.

Air cell administration of butylated hydroxy anisole in 96 hour embryos produced a significant increase in head and limb abnormalities in comparison with the absolute alcohol (solvent) controls. These data strongly suggest that butylated hydroxy anisole is teratogenic in low concentrations (1 and 2.5 mg/kg).

GENERAL PROCEDURES

The protocols as specified under FDA Contract #71-330 were followed in the investigation of toxicity and potential teratogenicity of the specified substance. The toxicity of the substance was evaluated from the percentage hatch of embryos injected either in the air cell or yolk at either zero hours (post-incubation) or after 96 hours incubation to provide four separate evaluations.

EGG SOURCE AND HANDLING

All eggs used in these investigations were from Shaver Starcross pullets housed at the Poultry Research Center of the University of Arizona in Tucson. The parent stock was maintained on the University of Arizona breeder diet which had been formulated to provide more than adequate amounts of all the known nutrients required by the breeding hen.

The feed was specially prepared to assure no contaminations and did not contain any additive drugs such as antibiotics. All eggs prior to use (within 48 hours of lay) were candled to remove any containing blood spots, abnormal air cells or abnormal shells, and only clean eggs ranging in weight from 23 - 26 ounces per dozen were used.

The supply flock was tested to assure the absence of Pullorum and Mycoplasma gallisepticum.

The eggs were incubated in forced draft Jamesway 252 machines with automatic temperature and humidity controls and an automatic turning device.

COMPOUND HANDLING FOR INJECTION

The substance tested was solubilized in a number of the prescribed solvents in order to determine the maximum concentrations which could be employed. Where possible, water was the solvent of choice. Maximum

injection volume was 0.05 ml. and all solvents and glassware were autoclaved prior to preparation of the solutions for use. The dose levels were administered with a microliter syringe using sterilized needles.

The preliminary range-finding studies using each of the administration routes and times were carried out with 10 - 25 eggs per dose level and included solvent controls, untreated controls and either drilled or pierced controls.

The actual dose-response protocol was carried out in two or more injections on different days to produce a minimum of 100 eggs at each dose level in five or more levels selected from the range- finding studies.

EXAMINATIONS OF EMBRYOS AND CHICKS

Eggs were candled daily and the dead embryos removed, examined and any abnormalities recorded. Five chicks from each dose level in each hatch were X-rayed to determine any skeletal abnormalities. Additional eggs injected at the approximate LD-50 level and an additional level below that were incubated and embryos at 8, 14, 17 days and hatch chicks removed for histopathological examinations.

In additional studies representative chicks from the dose-response protocol were saved. These chicks were housed in electrically-heated battery brooders with raised wire floors and fed University of Arizona diets. Feed consumption and growth rates were evaluated at 6 weeks of age and a sample of the birds sacrificed for gross and histopathological examinations.

The remaining birds in each group were maintained to 6 months of age and then sacrificed.

DATA HANDLING

All data were coded on forms provided by FDA for computer input. In addition to summaries of mortalities and abnormalities, a number of statistical evaluations were carried out. These statistical analyses included the following for both mortality and the incidence of abnormal embryos:

1. Chi-square tests for all dose levels and for each level against the solvent control.
2. Linear regression analyses + chi square test of linearity.
 - a. % response against dose
 - b. % response against log dose
 - c. log % response against dose
 - d. arcsin transformation against dose
 - e. arcsin transformation against log dose
3. Log dose against Probit using Finney's maximum likelihood method.
 - a. Where significant, the LD-30, 50, 70 and 90's were estimated with 95% confidence intervals.
4. One-way analyses of variance.
5. Linear regression with replication.

Butylated Hydroxy Anisole (71-74) solubilized in absolute alcohol for use in the test protocols. The maximum dose level employed was attained with a solution of 200 mg per ml to provide 200 milligrams per kilogram (10 mg/egg).

MORTALITY

The mortality data are shown in Tables 1 - 4 and exhibit 100% mortality with a 200 ppm dose with air cell administration at zero hours, while one-tenth of this dose level (20 ppm) produced 100% mortality when the air cell administration route was employed for embryos after 96 hours incubation. Much the same mortality pattern was obtained with yolk administration routes. The level of 200 ppm produced 83% mortality while a level of 25 ppm in the yolk at 96 hours produced 88% mortality of embryos.

Chi-square analyses of the mortality data are shown in Table 5. These calculations indicate that 25 ppm (mg/kg) of butylated hydroxy anisole and higher levels produced significant increase in embryo mortality when these amounts were injected into the air cell prior to incubation. When 96 hour embryos were employed using the air cell administration route, a level of 2 mg per kg produced a significant increase in mortality in comparison with the absolute alcohol solvent controls. Higher levels also produced significant increases in embryo mortality.

When the yolk administration route was employed with embryos after 96 hours incubation a level of 5 mg/kg and above produced significant increases in mortality (Table 5). Yolk administration of

either 100 or 200 mg butylated hydroxy anisole per kilogram produced significant increases in mortality. However, lower levels were not effective in this regard (Table 5).

Statistical evaluations employing linear regression analyses of log dose against probit of mortality indicate a significant relationship for three of the protocols (Table 6). A significant linear regression of log dose against probit of mortality was not obtained with the air cell administration route at zero hours (Table 6). The LD-50 estimates indicate that butylated hydroxy anisole was much more toxic after 96 hours incubation than when administered at zero hours. Employing the air cell administration route in 96 hour embryos produced an LD-50 estimate of 1.9 mg/kg for butylated hydroxy anisole, while yolk administration at zero hours produced an LD-50 estimate of 75.5 mg/kg, and employing the yolk administration route after 96 hours incubation the LD-50 was 8 mg/kg (Table 6). Butylated hydroxy anisole was toxic to chicken embryos when administered in either the yolk or cell.

TERATOLOGY

The one striking effect of butylated hydroxy anisole was the production of hypopigmentation in hatched chicks. This abnormality occurred when 25 ppm was administered in the air cell at zero hours incubation and in all embryos injected in the yolk at zero hours (Table 1 and 3). Yolk administration (96 hours) of 5 mg/kg also produced a high incidence of hypopigmentation (Table 4). Numbers of head, skeletal, visceral, and limb abnormalities recorded in Tables 1 through 4 were relatively low. The statistical significance of the hypopigmentation may be seen in Table 7.

A significant Chi-square for all abnormalities was obtained with the 25 mg/kg dose level using air cell administration at zero hours and for all dose levels above 10 mg/kg when yolk administration was used at zero hours.

Statistical evaluations of abnormalities other than the toxic response (hypopigmentation) indicate a significant increase in abnormalities of the head, limb, skeletal and visceral varieties for 1 mg/kg and 2.5 mg/kg administered in the air cell after 96 hours incubation. The incidence of these abnormalities were 7.18% at the 1 mg/kg level and 11.66% at the 2.5 mg/kg level (Table 2). These findings involved both the heads and limbs and are enumerated in Table 9.

These findings suggest that butylated hydroxy anisole may be teratogenic when administered at the lower dose levels in the air cell of fertilized eggs after 96 hours of incubation.

POST-HATCH DATA

The chicks maintained for six months post-hatch failed to show a significant effect of the administration of butylated hydroxy anisole during the incubation period. There were no significant alterations in growth rate or feed consumption or in sexual maturity in comparison with the absolute alcohol solvent control (Table 10).

TABLE 1
BUTYLATED HYDROXY ANISOLE in DEHYDRATED ALCOHOL
AIR CELL - 0 HRS

Dose, ppm	No. Fertile	Mortality % #		Abnormal Total % #		H-S-V-L % #		Abnormalities by category										
								Head % #		Skeletal % #		Viscera % #		Limbs % #		Struc- tural % #		Toxic Response % #
200.0	97	100.00	97	0.00	0	0.00	0											
100.0	128	70.31	90	5.46	7	0.00	0								5.46	7		
50.0	47	65.95	31	4.25	2	0.00	0								2.12	1		
25.0	98	20.40	20	46.93	46	3.06	3	2.04	2	1.02	1				43.87	43		
10.0	100	15.00	15	1.00	1	1.00	1	1.00	1									
5.0	60	6.66	4	0.00	0	0.00	0											
3.0	41	78.04	32	4.87	2	4.87	2					2.43	1	2.43	1			
0.5	40	0.00	0	0.00	0	0.00	0											
0.0	121	9.91	12	0.82	1	0.00	0								0.82	1		
drilled	220	10.45	23	0.00	0	0.00	0											
cont	734	4.49	33	0.54	4	0.54	4	0.40	3	0.13	1			0.13	1			

SUMMARY - ALL DOSE LEVELS

611	47.30	289	9.49	58	0.98	6	0.49	3	0.16	1	0.16	1	0.16	1		8.34	51	
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TABLE 2

BUTYLATED HYDROXY ANISOLE in DEHYDRATED ALCOHOL

AIR CELL - 96 HRS

Dose, ppm	No. Fertile	Mortality % #		Abnormal Total % #		H-S-V-L % #		Abnormalities by category							
								Head % #	Skeletal % #	Viscera % #	Limbs % #	Struc- tural % #	Toxic Response % #	Functional % #	
20.0	60	100.00	60	0.00	0	0.00	0								
15.0	60	98.33	59	0.00	0	0.00	0								
10.0	116	90.51	105	0.00	0	0.00	0								
5.0	175	91.42	160	3.42	6	5.14	9	3.42	6	0.57	1	1.14	2		
4.0	115	87.82	101	4.34	5	8.69	10	2.60	3	0.86	1	1.73	2	3.47	4
2.5	60	45.00	27	8.33	5	11.66	7	5.00	3			6.66	4		
2.0	164	66.46	109	5.48	9	5.48	9	1.21	2	0.60	1	3.65	6	0.60	1
1.0	167	46.70	78	5.98	10	7.18	12	2.99	5	0.59	1	1.19	2	2.39	4
0.2	60	28.33	17	1.66	1	0.00	0					1.66	1		
0.0	178	36.93	65	0.56	1	0.56	1			0.56	1				
drilled	254	4.33	11	0.39	1	0.39	1	0.39	1						
control	734	4.49	33	0.54	4	0.54	4	0.40	3	0.13	1		0.13	1	

SUMMARY - ALL DOSE LEVELS

977	73.29	716	3.68	36	4.91	48	1.94	19	0.20	2	0.61	6	2.05	20	0.41	0.00	0	0.00	0
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TABLE 3
BUTYLATED HYDROXY ANISOLE in DEHYDRATED ALCOHOL
YOLK - 0 HRS

Dose, ppm	No. Fertile	Mortality % #	Abnormal		Abnormalities by category							
			Total % #	H-S-V-L % #	Head % #	Skeletal % #	Viscera % #	Limbs % #	Struc- tural % #	Toxic Response % #	Functional % #	
200.0	150	93.33 140	6.66 10	0.00 0							6.66 10	
100.0	124	67.74 84	35.48 44	2.41 3	0.80 1				1.61 2		33.06 41	
50.0	19	15.78 3	84.21 16	0.00 0							84.21 16	
40.0	105	36.19 38	64.76 68	0.00 0						0.95 1	63.80 67	
25.0	20	10.00 2	90.00 18	0.00 0							90.00 18	
10.0	107	37.38 40	67.28 72	3.73 4	0.93 1			2.80 3			63.55 68	
5.0	17	17.64 3	0.00 0	0.00 0								
1.0	106	26.41 28	3.77 4	5.66 6	1.88 2	0.94 1	0.94 1	1.88 2				
0.0	126	35.71 45	0.79 1	1.58 2	0.79 1				0.79 1			
pierced	273	59.34 162	0.73 2	0.36 1	0.36 1					0.36 1		
control	734	4.49 33	0.54 4	0.54 4	0.40 3	0.13 1				0.13 1		

SUMMARY - ALL DOSE LEVELS

648	52.16 338	35.80 232	2.01 13	0.62 4	0.15 1	0.62 4	0.62 4	0.15 1	33.95 220	0.00 0
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Table 4

BUTYLATED HYDROXY ANISOLE in DEHYDRATED ALCOHOL

YOLK - 96 HRS.

Dose, ppm	No. Fertile	Mortality % #		Abnormal Total % #		H-S-V-L % #		Abnormalities by category						Toxic Response % #		Functiona % #	
								Head % #	Skeletal % #	Viscera % #	Limbs % #	Struc- tural % #					
25.0	102	88.23	90	0.98	1	0.00	0							0.98	1		
20.0	100	79.00	79	1.00	1	1.00	1			1.00	1						
10.0	100	55.00	55	6.00	6	0.00	0							6.00	6		
5.0	161	26.08	42	33.54	54	1.24	2	0.62	1	0.62	1			32.29	52		
4.0	60	15.00	9	0.00	0	0.00	0										
2.0	160	16.87	27	1.25	2	1.87	3	1.25	2	0.62	1						
1.0	60	23.33	14	1.66	1	1.66	1	1.66	1								
0.2	60	13.33	8	1.66	1	0.00	0						1.66	1			
0.0	161	13.66	22	0.00	0	0.00	0										
pierced	178	9.55	17	0.00	0	0.00	0										
control	734	4.49	33	0.54	4	0.54	4	0.40	3	0.13	1			0.13	1		

SUMMARY - ALL DOSE LEVELS

803	40.35	324	8.22	66	0.87	7	0.50	4	0.00	0	0.37	3	0.00	0	0.12	1	7.35	59	0.00
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TABLE 5
BUTYLATED HYDROXY ANISOLE
CHI-SQUARE ANALYSES of MORTALITY

DOSE LEVEL mg/kg	Air Cell		Yolk	
	0 hrs	96 hrs	0 hrs	96 hrs
0.2	-	1.11	-	0.03
0.5	2.97	-	-	-
1.0	-	2.98	1.90	2.33
2.0	-	28.46*	-	0.42
2.5	-	0.91	-	-
3.0	68.45*	-	-	-
4.0	-	71.46*	-	0.00
5.0	0.20	110.90*	1.46	7.04*
10.0	0.89	80.35*	0.02	48.71*
15.0	-	65.21*	-	-
20.0	-	68.93*	-	108.26*
25.0	3.97*	-	4.12	138.98*
40.0	-	-	0.00	-
50.0	52.92*	-	2.13	-
100.0	91.34*	-	24.40*	-
200.0	171.18*	-	100.28*	-
All doses (DF)	370.57*(8)	315.17*(8)	205.72*	313.29*(8)

*Probability > 0.05 - 0.005

Table 6
PROBIT ANALYSES
(LOG DOSE AGAINST PROBIT OF MORTALITY)

	Air Cell		Yolk	
	0 hrs	96 hrs	0 hrs	96 hrs
LD-30 (RANGE)	NS	0.9 (0.2 - 1.7)	75.8 (48.2 - 94.2)	7.2 (5.3 - 8.9)
LD-50 (RANGE)	NS	1.9 (0.8 - 2.9)	75.5 (75.5 - 119.6)	10.8 (8.8 - 13.1)
LD-70 (RANGE)	NS	3.7 (2.3 - 6.5)	133.7 (111.4 - 161.1)	16.2 (13.4 - 20.7)
LD-90 (RANGE)	NS	9.8 (5.7 - 39.2)	201.4 (166.0 - 291.7)	29.1 (22.5 - 44.3)

TABLE 7
BUTYLATED HYDROXY ANISOLE
CHI-SQUARE ANALYSES of ABNORMALITIES

DOSE LEVEL mg/kg	Air Cell		Yolk	
	0 hrs	96 hrs	0 hrs	96 hrs
0.2	-	0.0	-	0.27
0.5	0.34	-	-	-
1.0	-	6.46*	1.22	0.27
2.0	-	5.58*	-	0.51
2.5	-	7.98*	-	-
3.0	0.99	-	-	0.0
4.0	-	3.23	-	0.0
5.0	0.13	0.04	1.40	62.50*
10.0	0.33	-	115.85*	7.40*
15.0	-	0.32	-	-
20.0	-	0.32	-	0.06
25.0	65.60*	-	113.58*	0.05
40.0	-	-	108.84*	-
50.0	0.74	-	103.10*	-
100.0	2.95	-	48.63*	-
200.0	0.01	-	4.73*	-
All doses (DF)	235.08(8)*	24.71(9)*	325.35(8)*	220.00(8)*

*Probability > 0.05 - 0.005

TABLE 8

BUTYLATED HYDROXY ANISOLE
CHI-SQUARE ANALYSES of H-L-S-V ABNORMALITIES

DOSE LEVEL mg/kg	Air Cell		Yolk	
	0 hrs	96 hrs	0 hrs	96 hrs
0.2	-	0.32	-	0.0
0.5	0.0	-	-	-
1.0	-	4.44*	1.22	0.27
2.0	-	3.58	-	0.51
2.5	-	7.98*	-	-
3.0	0.33	-	-	-
4.0	-	3.23	-	0.0
5.0	0.0	2.36	1.40	0.50
10.0	0.01	0.04	1.20	0.0
15.0	-	0.32	-	-
20.0	-	0.32	-	0.10
25.0	0.75	-	1.12	0.0
40.0	-	-	0.01	-
50.0	0.0	-	1.20	-
100.0	0.0	-	0.27	-
200.0	0.0	-	0.01	-
All doses (DF)	9.816(8)	23.694(9)*	12.772(8)	6.332(8)

*Probability > 0.05 - 0.005

TERATOGENIC FINDINGS

TREATMENT	TOTAL NO. EXAMINED	TOTAL NO. ABNORMAL	TERATOGENIC FINDINGS	
			NO.	SPECIFIC FINDINGS
UNTREATED CONTROLS	734	4	1	microphthalmia-left, anophthalmia-right
			1	exencephaly, agenesia-maxilla
			1	dwarfism
			1	anophthalmia-bilateral, microcephaly, acrania, shortened maxilla, flexion-mandible, sclerostis-spinal nerve, agenesia, neck.
Drilled Control - 0 hrs	220	0	0	
Drilled Control - 96 hrs	254	1	1	anophthalmia-right, exencephaly, dysgnathia-beak
Pierced Control - 0 hrs	273	2	1	microphthalmia-right, shortened maxilla
			1	hypopigmentation
Pierced control - 96 hrs	178	0	0	
Butylated Hydroxy Anisole in alcohol - dehydrated				
Air Cell - 0 hrs				
200.0 mg/kg	97	0	0	
100.0	128	7	7	hypopigmentation
50.0	47	2	1	hypopigmentation
			1	hemorrhage - kidney

Sht. 2

TREATMENT	TOTAL NO. EXAMINED	TOTAL NO. ABNORMAL	TERATOGENIC FINDINGS													
			NO.	SPECIFIC FINDINGS												
				D	E	S	C	R	I	P	T	I	O	N		
25.0	98	46	1	hemorrhage-umbilical cord												
			1	anophthalmia-right, dysgnathia												
			1	microphthalmia-left, dysgnathia, torticollis-neck												
			43	hypopigmentation												
10.0	100	1	1	anophthalmia-bilateral, dysgnathia												
5.0	60	0	0													
3.0	41	2	1	ectromelia-right wing, celosomia												
			1	hemorrhage-kidney												
0.5	40	0	0													
0.0		1	1	hemorrhage - umbilical chord												
BHA in air cell - 96 hrs.	121															
20.0 mg/kg	60	0	0													
15.0	60	0	0													
10.0	116	0	0													
5.0	175	6	1	acrania, ectromelia - right wing												
			1	buphthalmia - right												
			1	anophthalmia-right												
			1	microphthalmia-left, exencephaly, fusion failure-skull												
			1	agenesis-right wing, celosomia												
			1	anophthalmia-left, exencephaly, dysgnathia												
			1	fusion failure - skull												

TABLE 9

Sht. 4

[illegible]

Sht. 5

Sht. 5

TREATMENT	TOTAL NO. EXAMINED	TOTAL NO. ABNORMAL		SPECIFIC FINDINGS
			NO.	D E S C R I P T I O N
Butylated Hydroxy Anisole in Alcohol-Dehydrated				
Yolk - 0 hrs				
200.0 mg/kg	150	10	10	hypopigmentation
100.0	124	44	44	hypopigmentation
			1	malrotation-left hind limb
			1	dysgnathia
			1	ankylosis-bilateral
50.0	19	16	16	hypopigmentation
40.0	105	68	67	hypopigmentation
			1	dwarfism
25.0	20	18	18	hypopigmentation
10.0	107	72	1	anophthalmia-left, brachygnathia mandible
			3,	celosomia
			68	hypopigmentation
5.0	17	0	0	

Sht. 6

[illegible]

Sht. 7

[illegible]

TABLE 10
BUTYLATED HYDROXY ANISOLE
POST HATCH DATA

Label	Dose mg/kg	Age at Sexual Mortality	at Hatch	Average Body Wt., gm				Average Feed Consumption per Bird	
				6 wks		6 mos		6 wks, gm	6 mos, kg
				M	F	M	F		
203	25.0	150	39.8	406	355	1702	1625	942	10.25
205	5.0	148	40.2	427	391	1702	1476	1010	9.81
206	ETOH	147	38.8	429	423	1698	1553	961	10.21